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Immobilization of a bone and cartilage stimulating peptide to a synthetic bone graft

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Abstract A synthetic peptide fragment of human collagen type I (BCSP^{TM}-1) was linked to the surface of a commercially available ceramic in an effort to improve the properties of the bone graft substitute to accelerate local healing. BCSP^{TM}-1 was covalently immobilized on the surface of the ceramic via the linkers 3-aminopropyltriethoxysilane (APTES) and suberic acid bis-*N*-hydroxysuccinimide ester (DSS). The chosen chemistry was non-cytotoxic. A rat calvaria cell assay using alkaline phosphatase (ALP) as an osteoblast differentiation marker, showed that modifying the surface of the ceramic was enough to enhance ALP activity, although the total cell population on the surface decreased. A significant increase in ALP activity/cell was noted with serum albumin bound to the surface, however, the BCSP^{TM}-1 bound surface exhibited an even greater ALP activity that showed a surface concentration dependent trend. An optimal BCSP^{TM}-1 surface density in the range of 0.87–2.24 nmol/cm^2 elicited the maximum ALP activity/cell at day 6 of culture. The peptide bound ceramic generated an ALP activity/cell that was roughly 3-fold higher than the non-modified ceramic and 2-fold higher than the APTES-grafted ceramic.

1 Introduction

Large bone defects resulting from trauma, tumors, infections, or congenital abnormalities often require reconstructive surgery to restore bone function. Currently there are about 500,000 bone graft surgeries performed annually in the United States, with the estimated cost of these procedures approaching $US 3 billion [1]. The majority of this market segment is comprised of naturally sourced grafts (~90%) with the balance consisting of synthetically sourced grafts (~10%) [2]. However, shortcomings associated with the usage of natural grafts, such as limited supply and immunologic restrictions, have stimulated interest in utilizing synthetic bone graft substitutes. In particular, orthopedic research has been focused on improving synthetic bone graft properties to make them comparable to naturally sourced grafts; that is, possessing both osteoconductive and osteoinductive properties.

Some of the most common synthetic bone substitute materials include calcium phosphate based ceramics, coral calcium carbonates, non-ceramic calcium phosphates in polymer form, and calcium sulfates [1–3]. These synthetic biomaterials all possess excellent biocompatibility, predictable biodegradability, and osteoconductive characteristics. The common drawback of these synthetic materials is that they do not have an equal ability as naturally sourced grafts to elicit osteoinductivity. Surface properties of a ceramic bone substitute are only part of the solution, as other features must be considered such as the ability to bear weight immediately after implantation and handling properties in the operating arena. However, early osteointegration of the implant with the host tissue is recognized as being key to preventing the need for later surgical intervention.

One approach that has been examined to improve the osteoinductivity of ceramic materials, is the binding of peptides or proteins to their surface covalently or via physical adsorption. A common covalent binding method involves first grafting a silane group to the hydroxyl groups available at the surface followed by coupling a peptide to

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the silane group through a bifunctional crosslinker [4–7]. Hydroxyapatite is also highly effective at physically adsorbing peptides and proteins [8–15]. With physical adsorption, the bioactive molecules are held by weak van der Waals forces and/or ionic interactions and are randomly dispersed. The orientation of the adsorbed molecule is difficult to control, and this methodology leads to large inconsistencies in amount of peptide at the surface. Adsorbed molecules are not guaranteed to elicit specific cellular responses and their reactive sites may or may not be available. In contrast, chemical modification results in irreversible binding, with high levels of surface coverage, and avoids the delamination and desorption of bioactive molecules that is commonly encountered with the physicosorption method. In addition, it is easier to control the concentration and the orientation of attached bioactive molecules via covalent attachment. When molecules are bound onto the surface, they are retained on the surface until acted upon by ligand interactions and/or cellular activities. Other potential advantages include a longer retention time for immobilized molecules and the option to release immobilized molecules when desired (e.g. cleavable crosslinkers) [16].

The peptide most studied for surface modification has been the arginine-glycine-aspartic acid (RGD) sequence. In the studies done thus far with RGD bound to hydroxyapatite surfaces, most have focused on the ability of the RGD sequence to improve cell attachment and spreading on the surface. Recent studies have demonstrated that osteoblastic and osteoprogenitor cells attach to RGD grafted hydroxyapatite in a concentration dependent manner. Both Itoh et al. [12] and Sawyer et al. [11] found that attached cell populations on RGD containing surfaces increased as the amount of RGD on the surface increased, reaching a maximum at between 5 and 10 μg/mL of RGD in the surface treatment medium, then decreased thereafter for human osteoprogenitor cells [11] or remained constant for osteoblastic cells [12]. However, few have examined whether the bound RGD induces the differentiation of the osteoprogenitor cells into osteoblasts. Itoh et al. [12] found that markers of osteoblast activity, such as alkaline phosphatase activity and expression of bone matrix proteins, peaked at concentrations of poly(glutamate) conjugated RGD in the surface treatment medium of between 5 and 10 μg/mL, in conjunction with the peak concentrations for osteoblastic cell adhesion. However, in a recent study by Sawyer et al., in which RGD was adsorbed to a hydroxyapatite surface in a similar fashion, it was concluded that RGD-coated hydroxyapatite was not able to induce mesenchymal stem cell spreading, which is necessary for cell survival and osteoblastic differentiation, and that hydroxyapatite coated with serum provided better results [17]. Thus, alternative approaches to coating with RGD appear necessary.

In this work, we develop a method for covalently attaching peptides to the surface of Skelite™ a synthetic bone graft material, verify the chemistry used, and examine the ability of a bone and cartilage stimulating peptide, or BCSP™-1, covalently immobilized to the surface of Skelite™, to attach osteoprogenitor cells from a fetal rat calvaria cell pool as a function of the peptide surface density. Skelite™ is a biodegradable ceramic composed of 33% hydroxyapatite (HA) and 67% silicon-stabilized tricalcium phosphate (Si-TCP) [18, 19]. BCSP™-1 is a synthetic peptide fragment of human collagen type I, composed of an Asn-Gly-Leu-Pro-Gly-Pro-Ile-Gly-hydroxy Pro amino acid sequence (NGLPGPIGP) and having a molecular weight of 835 g/mol. Matrices of collagen type I have been demonstrated to enhance proliferation and accelerate differentiation of osteoblastic cells [20, 21]. Similarly, BCSP™-1 has been shown to promote growth activities in bone and cartilage [22]. As a synthetic fragment of collagen type I, the potential advantage of BCSP™-1 is that it stimulates growth while eliminating the possibility of disease transmission inherent in using biologically derived collagens.

2 Materials and methods

BCSP™-1 and Skelite™ discs were provided by Millenium Biologix Inc. The dimensions of the Skelite™ discs were 12 mm in outer diameter and 2 mm in thickness. These discs consist of pressed Skelite™ powder, and possess a non-porous surface. The Skelite™ discs were washed with anhydrous ethanol and dried in a vacuum oven prior to use. The peptide was received as a lyophilized powder and stored at −20 °C before use. The surface linkers 3-aminopropyl-triethoxysilane (APTES) and suberic acid bis-*N*-hydroxysuccinimide ester (disuccinimidyl suberate or DSS), and the solvents dimethylformide (DMF) and anhydrous ethanol (EtOH) were purchased from Sigma, Canada and used as received.

BCSP™-1 immobilization onto the surface of Skelite was achieved by means of a three-step reaction procedure (Fig. 1). The first step was a silanization reaction involving a covalent attachment of APTES grafted to Skelite™ via free hydroxyl groups to introduce primary amine moieties on the surface. The second step was a succinylation reaction involving the formation of a covalent amide bond by a substitution of amine functionality (APTES) with a succinimide ester group contributed by suberic acid bis-*N*-hydroxysuccinimide ester (DSS). Lastly, BCSP™-1 grafting was achieved via a stable covalent amide bond by the substitution of its N-terminus with a succinimide ester

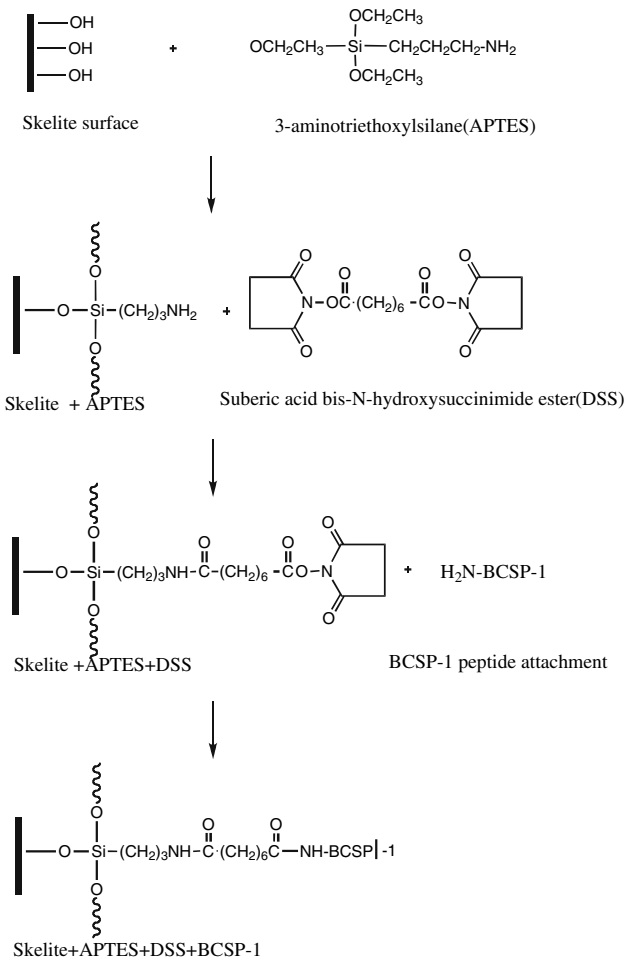

Fig. 1 Chemistry of BCSP-1 peptide covalent linking to Skelite ceramic surface employing APTES and DSS

group from the grafted suberic acid bis-*N*-hydroxy-succinimide ester.

2.1 Surface functionalization

A solution of 10% APTES in anhydrous ethanol was prepared and used to add 500 mM of APTES to each disc/well in a 24-well plate. The reaction was allowed to proceed for 2 h at ambient conditions. The reaction medium was quenched by rinsing the surface three times with 2 mL of anhydrous ethanol and the disc was allowed to air dry within the laminar flow hood for 2 h prior to the attachment of DSS. 25 mM of DSS dissolved in 2:3 v/v DMF:EtOH was added to each silanized Skelite^{TM} sample. The reaction was allowed to proceed for 4 h at ambient conditions. The reaction was quenched by washing the surface three times with 2 mL of anhydrous ethanol and the disc was rinsed with sterilized de-ionized water prior to peptide attachment.

2.2 Covalent peptide attachment

BCSP^{TM}-1 was dissolved in de-ionized water and syringe filtered prior to incubation with the surface functionalized Skelite at room temperature for 48 h. A total of five different initial dosage concentrations of BCSP^{TM}-1 (558, 212, 58, 5.8 and 0.58 nmol/cm^2) were chosen to determine the surface density dependence and bioactivity of the bound peptide. Following peptide grafting, the samples were washed 5 times with sterilized de-ionized water to remove unreacted peptide and hydrolyze unreacted *N*-hydroxysuccinimide ester groups, and allowed to air dry within the laminar flow hood. Bovine serum albumin (BSA) was also linked onto the surface and used as a comparison to the BCSP^{TM}-1 bound surface. High Performance Liquid Chromatography (HPLC) was employed to determine the amount of BCSP^{TM}-1 attachment. Each BCSP^{TM}-1 concentration (above) was allowed to react with a DSS-grafted surface and the residual concentration in the reaction medium was determined. The difference between the two concentrations was taken to be that of the attached peptide. A bicinchoninic acid (BCA) protein assay from Pierce was utilized to determine the amount of BSA immobilized on Skelite^{TM} surface.

2.3 Adsorption of BCSP^{TM}-1 to Skelite

BCSP^{TM}-1 was dissolved in de-ionized water to a concentration of 66 nmol/mL and syringe filtered. One mL of this solution was added to the discs (Skelite^{TM} alone and Skelite^{TM} reacted with APTES) placed within wells in a 24-well plate. The discs were incubated in this solution for 72 h, after which they were washed twice with sterilized de-ionized water. The amount of BCSP^{TM}-1 adsorbed to each disc was determined using the HPLC assay method.

2.4 BCSP^{TM}-1 HPLC assay

A Waters (Milford, MA, USA) chromatographic system was used, composed of a model 600 solvent pump, a model 717 auto injector, and a model 474 scanning fluorescence detector with excitation and emission wavelengths set at 488 nm and 512 nm. A 15 cm × 4.6 mm inner diameter Kingsorb C18 analytical column packed with 3 μm particle size and a pre-column insert packed with C18 (Phenomenex, Torrance, CA, USA) were used for separation. The mobile phase consisted of 80% acetonitrile, 19.9% distilled de-ionized water and 0.1% trifluoroacetic anhydride. The stationary phase consisted of 10% acetonitrile, 89.9% distilled de-ionized water and 0.1% trifluoroacetic anhydride.

2.5 Surface characterization

The surface chemistry efficacy was confirmed utilizing X-ray photoelectron spectroscopy (XPS), and Time of Flight Secondary Ion Mass Spectroscopy (TofSIMS). The XPS spectra were obtained on a Leybold MAX 200 X-ray photoelectron Spectrometer (LH, Cologne, Germany) utilizing an unmonochromatized magnesium K_α radiation source operating at 15 kV and 20 mA. The energy range was calibrated against Cu $2p_{3/2}$ and Cu 3p lines at 932.7 and 75.1 eV respectively. In addition, energy scales were referenced by placing the C 1s value for C–H/C–C component at 285 eV and data reduction was performed by applying PHI-access software. Absolute areas were calculated using the parabolic integration routine and background energy was calculated using the trapezoid area method. TofSIMS spectra were obtained using a Ga liquid metal gun, on the Ino-Tof Gmbh (Munster, Germany). Both positive and negative spectra were obtained using an 800 pA DC primary ion beam pulsed with a frequency of 8 kHz and a pulse width of 12 ns over a total surface area of 200 × 200 μm^2. The total integrated primary ion dose was $<10^{12}$ ions/cm^2.

2.6 Cell culture

Rat calvaria cells were cultured on surface modified Skelite™ samples and evaluated for osteoblastic activities at days 6 and 10 of culture. Passage 1 rat calvaria cells were harvested from a pool of 20–30 newborn rats. Using sterile procedures, 24–30 rat pups were euthanized with isofluorane (Bimeda-MTC). Cells were extracted by digesting collagen from the calvaria using 30 mL of Hank's Buffer, collagenase (Worthington), dispase I (Roche Diagnostics), and DNAse (Sigma). After digestion, the cell concentration was determined using a hemocytometer, and cells were seeded to cell culture flasks (NUNC T80) at a density of 5×10^5 cells per flask in 20 mL of cell culture medium. The culture medium consisted of α-MEM supplemented with 10% FBS, 0.3 μg/mL fungizone, and 0.28 mM L-ascorbic acid 2-phosphate. After 3 days of growth in the flasks, the cells were extracted using a solution of 0.05% trypsin (GIBCO) and 0.53 mM EDTA (GIBCO) at 37 °C. These cells were seeded on surface modified Skelite™ samples at a density of 12,000 cells per cm^2. Plates were placed in a tissue culture incubator at 37 °C with 5% CO_2. The culture media was changed every 48 h.

At day 6 or 10 of culture, cells were lysed to extract cell lysates for alkaline phosphatase (ALP) and DNA measurements. The supplemented medium was aspirated and washed once with PBS to remove any protein residue from the culture medium. About 500 μL of lysis buffer (Cellytic Mammalian Cell Lysis/Extraction Reagent, Sigma C2978) was added to each sample and the samples were incubated for 30 min at room temperature. Cells were scraped off the surface of the disc using a pipette tip and the extraction medium was transferred into Eppendorf test tubes. The Eppendorf tubes were then centrifuged at a rate of 13,000 rpm for 10 min. This extra step was carried out as a precautionary action to remove any ceramic debris that might have resulted from scraping the cells off the surface of the Skelite™ samples. The supernatant (extraction medium) was separated from the precipitate debris and used for analysis.

The level of alkaline phosphatase (ALP) activity in the culture medium was measured as follows. About 50 μL of the alkaline phosphatase buffer (Sigma) was transferred to a Nunc 96-well plate, followed by the addition of 10 μL of lysed sample and 50 μL of phosphatase substrate (Sigma). Blanks were prepared to serve as background absorbance readings and subtracted from the sample readings. Alkaline phosphatase standards were prepared in concentrations ranging from 0 to 200 nM of *p*-nitrophenol from serial dilutions of 10 mM *p*-nitrophenol standard solution (Sigma) with 0.02 N NaOH. The standards and the blank solutions were added to the same 96-well plate along with the samples, shaken on a microplate incubator for several seconds to allow uniform mixture and incubated at 37 °C for 15 min. The colorimetric reaction was then quenched with the addition of 100 μL of 0.2 N NaOH to each well. The absorbance of each well was measured using a microplate reader at 405 nm and results were normalized to total DNA and expressed in nmol/mL of *p*-nitrophenol per time unit per ng/mL of DNA.

Total DNA quantification was determined to normalize ALP activity and to correlate the enzymatic activity on a per cell unit basis. In brief, 50 μL of the lysed sample was added to 50 μL of PicoGreen solution (dsDNA Quantitation Kit, Molecular Probes). Blanks were prepared utilizing the same protocol except swapping the sample solution with an equal amount of lysis buffer. Standards in concentrations ranging from 0 to 2000 ng/mL were prepared from serial dilutions of 100 μg/mL stock lambda DNA standard solution with 1× TE Buffer (10 mM Tris–HCl and 1 mM EDTA at a pH 7.5). Standard and blank solutions were added to the same 96-well plate. The assay plate was incubated in the dark for 5 min at room temperature and absorbance was measured at a fluorescence wavelength of 485 nm excitation and 538 nm emission.

2.7 Statistics

A one-way Anova analysis was performed to test statistical significance between different groups. The hypothesis that

the population means being compared were equal was rejected if the *p*-value was less than 0.05 (95% confidence). Day 6 BCSP^{TM}-1 data analyses were based on 20 replicates, while day 10 BCSP^{TM}-1 and all BSA bound surfaces data analyses were based on six replicates.

3 Results

3.1 Surface characterizations

Hydroxyl groups on the surface of the Skelite^{TM} are required to covalently bind a bioactive agent. However, it has been demonstrated that the presence of Si in the crystal lattice replaces the hydroxyl groups that may be available for chemical binding [18, 19]. The presence of a sufficient number of hydroxyl groups is necessary for effective surface modification. Confirmation of APTES linkage to the surface-available hydroxyl groups was therefore initially determined using XPS. All spectra were compared to a surface minus one layer of modification.

XPS spectra for non-modified Skelite^{TM} and APTES-grafted Skelite^{TM} are shown in Fig. 2a and b. The APTES-grafted surface had an increase in total atomic percentage of nitrogen from 0% to 3.7% and total carbon from 29.3% to 42%. The increase in nitrogen was due to the introduction of primary amine ($-NH_2$) on the surface and the increase in carbon was due to the alkaline chain dispersed by the APTES.

The C1s spectra of the Skelite^{TM} and surface grafted Skelite^{TM} are provided in Fig. 3a–c. Examination of the DSS-grafted surface indicates that the C peak associated with CH_2N, CH_2O (at 286.2 eV) increased from 14.3% to 21.2% (Fig. 3b). The increment in this elemental state indicates the presence of C–N bonds contributed by the succinimidyl group. In addition, increases were observed in two other carbon peaks in the form of O=C–N (288.0 eV) and O=C–O (289.3 eV) from 4.4% to 9.5% and 1.4% to

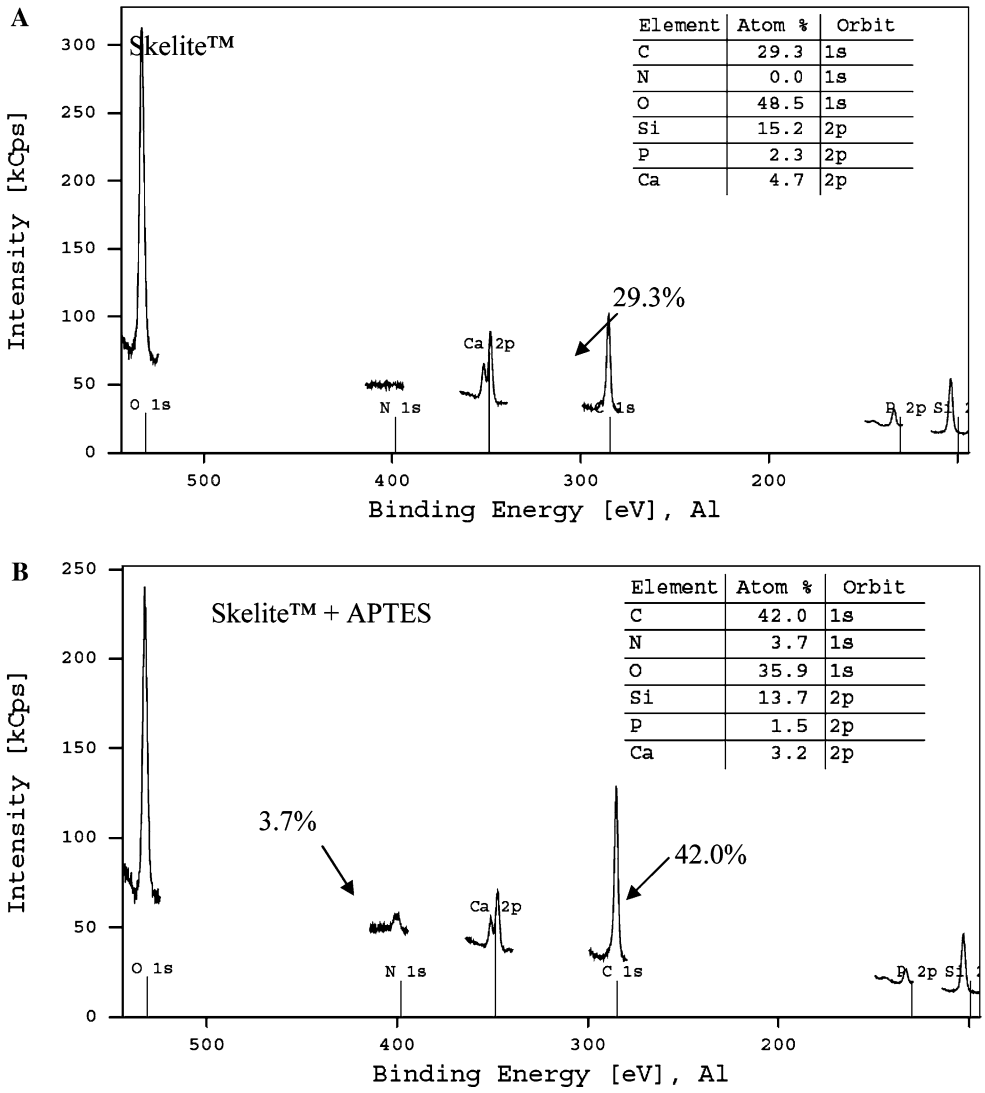

Fig. 2 XPS spectrum for Skelite^{TM} (**a**) and Skelite-APTES-grafted surface (**b**)

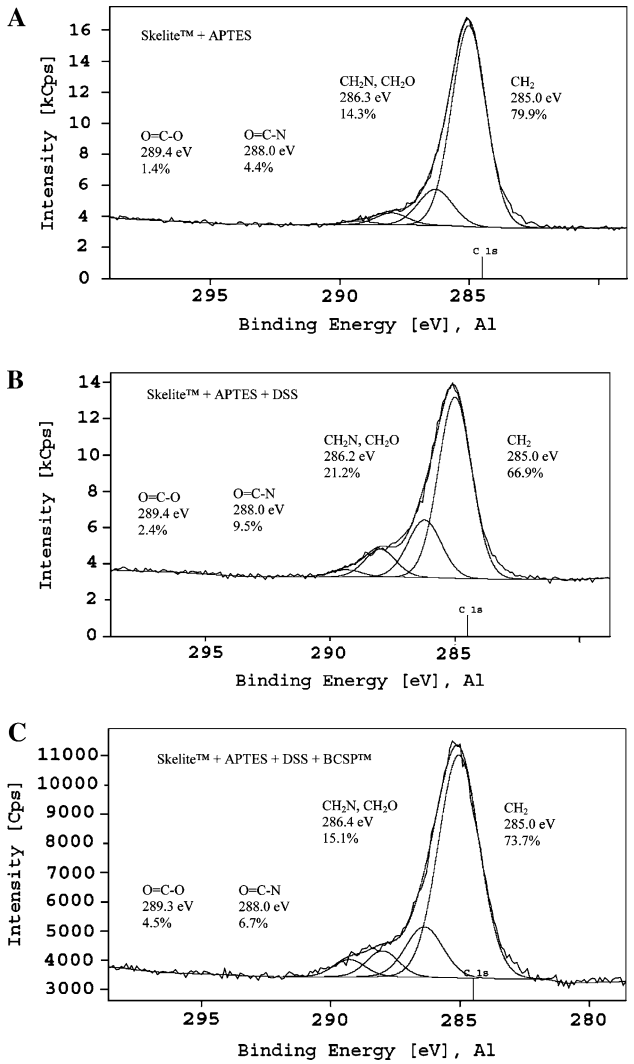

Fig. 3 (**a**) XPS C 1s spectrum for APTES-grafted surface, (**b**) XPS C 1s spectrum for DSS-grafted surface, and (**c**) XPS C 1s spectrum for BCSP^{TM}-1 bound surface

2.4% respectively. The increments in these carbon peaks are due to ester and amide bonds from the DSS. Examination of the XPS C1s peaks for the peptide bound surface and the DSS-grafted surface, demonstrated that the peak for the carbon in the form of CH_2 (284.9 eV) increased from 69.9% to 73.7% and the carboxyl carbon O=C–O (289.4 eV) increased from 2.4% to 4.5% (Fig. 3c). A summarized table of XPS spectra for all surfaces is listed in Table 1.

TofSIMS analysis was performed to confirm the peptide binding indicated by the XPS data. BCSP^{TM}-1 is a 9-mer amino acid (NGLPGPIGP) and was attached via its N-terminus (Asn terminal) to the Skelite^{TM} surface via APTES and DSS crosslinkers. Identified fragmentation masses from the peptide bound spectrum include: peptide bonds (O=C–NH^+ at *m/z* 43.15), amino acid residues (H_2N=CH–R^+ at *m/z* 28.90+R) and peptide fragments for glycine, proline, leucine and hydroxyproline. Asparagine was difficult to identify because its fragments were incorporated in the amino acids residues H_2N=CH–R^+. A complete list of the identified fragments is tabulated in Table 2.

3.2 Determination of surface density of bound BCSP^{TM}-1 and BSA

With the presence of BCSP^{TM}-1 on the surface confirmed, the extent of covalent binding of BCSP^{TM}-1 and BSA under the reaction conditions were determined. Values of investigated reaction concentrations and the corresponding peptide/BSA attachments are shown in Fig. 4. All residual concentrations ($n = 20$ for peptide and $n = 6$ for BSA) were significantly ($p < 0.05$) less than the initial dosage to support peptide/BSA attachment. The degree of attachment showed a concentration dependent trend reaching a maximum to indicate that a saturation concentration had been reached. At all initial concentrations, BSA was bound to a greater extent than the BCSP^{TM}-1 peptide.

3.3 In vitro assessments

3.3.1 Cell proliferation

The rat calvaria cells adhered and proliferated on all surfaces, indicating that the chosen chemistry was non-cytotoxic. After 6 days, the rat calvaria cells adhered and proliferated to a greater degree on the unmodified Skelite^{TM}

Table 1 XPS C 1s chemical state quantifications

	CH_2 (~285 eV) (%)	CH_2N, CH_2O (~286 eV) (%)	O=C–N (~288 eV) (%)	O=C–O (~289 eV) (%)
Skelite^{TM}	79.0	13.3	4.3	3.2
Skelite^{TM} + APTES	79.9	14.3	4.4	1.4
Skelite^{TM} + APTES + DSS	69.9	21.2	9.5	2.4
Skelite^{TM} + APTES + DSS + BCSP^{TM}	73.7	15.1	6.7	4.5

Expressed in atomic percentages and binding energies

Table 2 Percentage increase in correlated intensity for positive BCSP^{TM}-1 spectrum

MW	Molecular fragments	Intensity increase[a]
28.90	NH_2CH^+	13×
30.20	Glycine, amino acids residues ($H_2NCH_2^+$)	126×
43.15	Peptide bonds ($OCNH^+$)	26×
45.12	Carboxyl ($COOH^+$) or $^+HNCH_2CH_3$	147×
70.31	Proline ($C_4H_8N^+$)	3×
85.95	Leucine ($C_5H_{18}N^+$)	9×
131.80	Hydroxyproline ($C_5H_{10}ON^+$)	5×

[a] The amount of intensity increase was compared to DSS-graft Skelite^{TM} surfaces

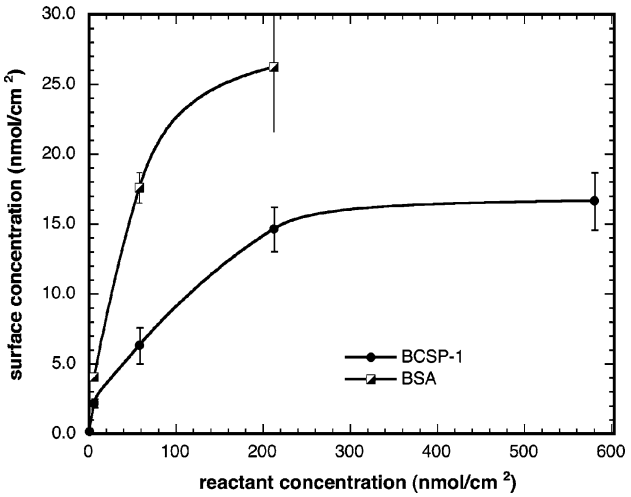

Fig. 4 Influence of BSA or BCSP-1 initial concentration on resulting bound surface density

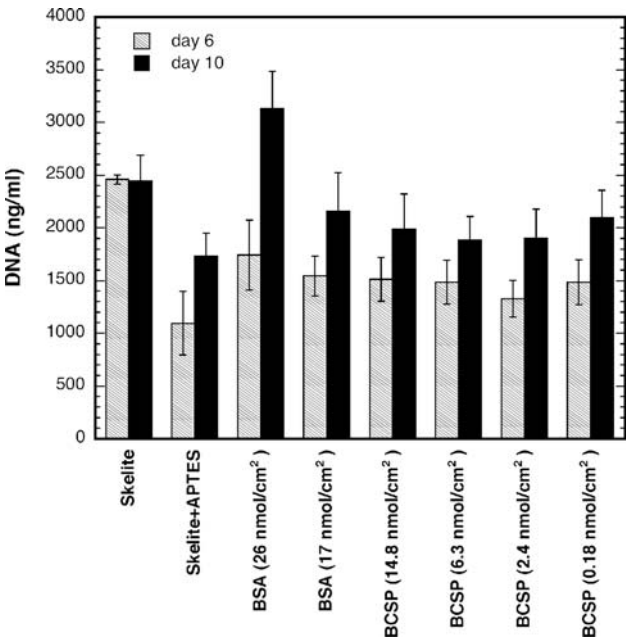

Fig. 5 Cell densities on differently treated ceramic substrates expressed in terms of total DNA concentration. BSA and BCSP-1 were immobilized onto the surface using the same chemistry and the substrate surface areas are the same in each case. The value in the brackets represents the surface density of immobilized BSA or BCSP-1

than to any other surface (Fig. 5). The APTES modified surface exhibited the lowest cell population (roughly 1/3 less than that of the unmodified Skelite^{TM}), while that of the BSA and BCSP^{TM}-1 immobilized surfaces were intermediate that of unmodified Skelite^{TM} and Skelite^{TM} with grafted APTES. After 10 days, the cell population on the Skelite^{TM} surface had remained essentially constant, while all other surfaces exhibited an increase in cell density. The APTES modified surface had the least average population density; however, it was statistically comparable to those of all other modified surfaces, with the exception of the surface containing 24 nmol/cm^2 of BSA which possessed a statistically significantly higher population of cells.

3.3.2 Alkaline phosphatase activity

In vivo, once osteoprogenitors become committed to the osteoblast lineage, there is a progressive increase in the expression of markers of differentiation, such as alkaline phosphatase (ALP) activity [23, 24]. ALP expression was measured at two time points to evaluate the osteoblast populations on the surface modified Skelite^{TM} samples. The results are given in Fig. 6a and b.

Despite the greater cell numbers, rat calvaria osteoprogenitor cells on the non-modified Skelite^{TM} samples expressed the least amount of ALP on a per cell basis (0.16 ± 0.02 nM PNP/min/ng DNA/mL) when compared to all other surface-modified samples at days 6 and 10 (Fig. 6a). There was no significant difference in ALP activity/cell for the Skelite^{TM} samples from day 6 to day 10. The ALP activity was significantly higher for the APTES modified Skelite^{TM} surfaces than the Skelite^{TM} surfaces at both time points, and again, there was no significant change in activity as time progressed. At day 6, the BSA modified surface had significantly higher ALP activity than the Skelite^{TM} and Skelite^{TM} + APTES surfaces for all surface densities examined; however, at day 10, the ALP activity/cell had significantly decreased and was comparable to that of the Skelite^{TM} and Skelite^{TM} + APTES surfaces. There was no influence of BSA surface density on the ALP activity/cell at either day 6, with ALP activities of 0.36 ± 0.06, 0.40 ± 0.02, and 0.44 ± 0.07 nM PNP/min/ng DNA/mL at surface densities of 4, 16, and 27 nmol/cm^2, respectively (Fig. 6b). The same surface density independent trend was observed at day 10.

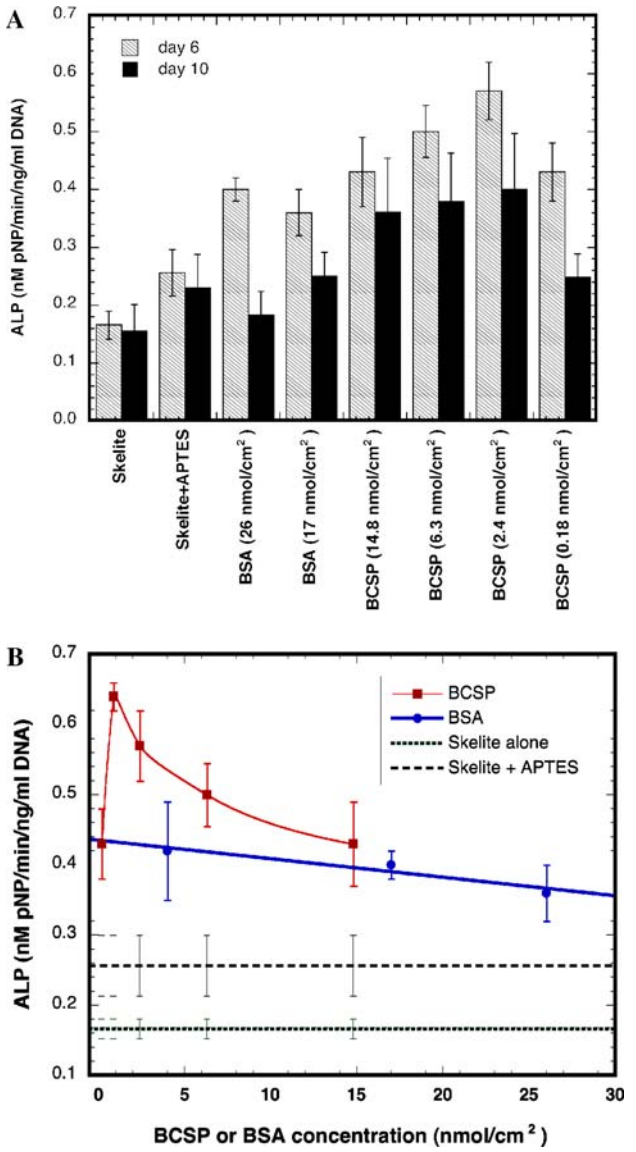

Fig. 6 Influence of concentration of immobilized BCSP-1 versus immobilized BSA on rat calvaria osteoprogenitor cell ALP activity. (**a**) Comparison of ALP activity/cell at days 6 and 10 of culture and (**b**) Highlight of concentration effect at day 6

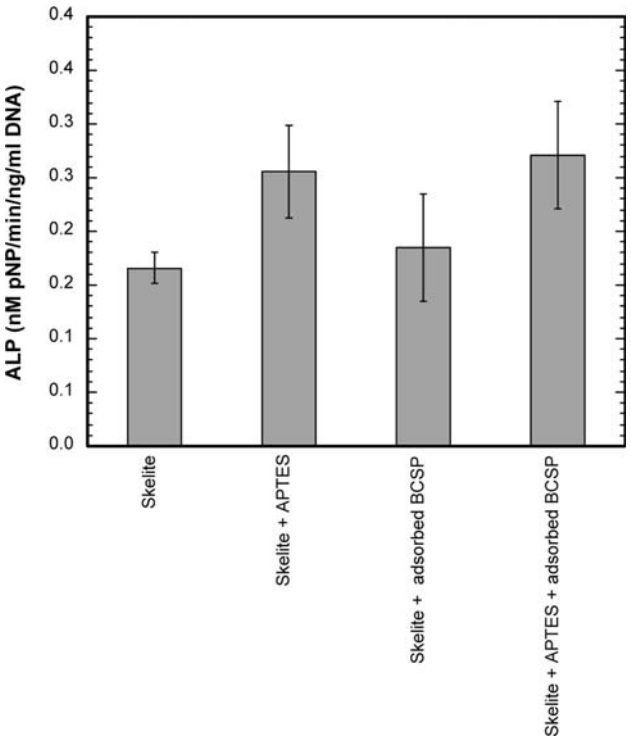

Fig. 7 Comparison of Skelite discs exposed or not exposed to BCSP-1 solution for adsorption on rat calvaria osteoblast ALP activity at day 6 of culture

BCSP^{TM}-1 bound surfaces, on the other hand, demonstrated a surface density dependent ALP activity/cell. At the lowest surface density of 0.18 ± 0.05 nmol/cm^2, the ALP activity was comparable to that of the BSA bound surfaces at day 6 and day 10. Maximum activity occurred between BCSP^{TM}-1 surface densities of 0.87–2.24 nmol/cm^2 at day 6. Activity levels fell after reaching this maximum and plateaued at surface densities greater than 14.8 nmol/cm^2 (Fig. 6b). At the higher surface densities, the ALP activity was again comparable to that of the BSA bound surface ($p > 0.05$). Although the average ALP activity/cell values were lower at day 10 than observed at day 6, there was no statistically significant difference in the averages at each time point ($p > 0.05$). However, at day 10, there was no BCSP^{TM}-1 surface density dependent trend of ALP activity/cell for BCSP^{TM}-1 surface densities greater than 0.18 nmol/cm^2.

BCSP^{TM}-1 was adsorbed onto a non-modified Skelite^{TM} and onto an APTES-grafted Skelite^{TM} and compared to the Skelite^{TM} having the BCSP^{TM}-1 covalently linked on the surface. All compared Skelite^{TM} samples were initially dosed at a concentration of 5.8 nmol/cm^2 and the same binding/washing protocol was followed. HPLC analysis of the wash solutions indicated that BCSP^{TM}-1 was not adsorbed to either the Skelite^{TM} or the APTES-grafted Skelite^{TM}. Both peptide-exposed surfaces exhibited ALP activities per cell that were statistically equal to that of the non-BCSP^{TM}-1 exposed surfaces, supporting the HPLC based analysis indicating that BCSP^{TM}-1 was not effectively adsorbed to the surfaces (Fig. 7).

4 Discussion

The XPS and Tof-SIMS analysis shows that the BCSP^{TM}-1 was successfully bound to the Skelite^{TM} surface, and that this binding could be accomplished in a surface concentration dependent manner. These increased percentages for carbon associated with CH_2N, CH_2O, O=C–N and O=C–O

indicate that DSS was successfully attached to the surface and that the increase in these different carbon forms are due to the presence of the ester and succinimide groups from the DSS. By comparing the XPS C1s peaks for the peptide bound surface and the DSS-grafted surface, it was observed that the peak for the carbon in the form of CH_2 (284.9 eV) has increased from 69.9% to 73.7% and the carboxyl carbon O=C–O (289.4 eV) increased from 2.4% to 4.5% (Fig. 3c). These are indications that extra carbons and carboxylic groups (from the peptide) have been added onto the surface. However, the carbons in the form of O=C–N experienced a decrease from 9.5% to 6.7%. Theoretically, for every two amide carbon O=C–N cleavage (from succinimide), there should be an additional eight peptide bonds added on the surface from the attachment of the 9-mer BCSP^{TM}-1. One explanation for this discrepancy could be due to signal attenuation produced by the coupling layers. In other words, the extra peptide layer attenuated XPS signals and not all possible elements were detected. One of the major limitations of XPS is that its surface depth profiling is poor [25]. In addition, a non-monochromatic electron source was used and resulted in a broader and less penetrating analysis area, thereby reducing the signals. In a related study by Zhang et al. [26] a decrease in the O=C–N C 1s XPS spectrum after GRGDSY peptide attachment to a silica surface via a succinimidyl crosslinker was also observed. This decrease for the carbon O=C–N was also attributed to an attenuated XPS signal. Approximately 6.7% of total carbons are present as amide bonds, yet the theoretical percentage of amide bonds was calculated to be in the range of 23–25% (12 amide carbons per 52, 50, and 48 total carbons for first, second, and third coupling degrees of APTES). Since the experimental value was almost a third of the theoretical, the observation can be interpreted as a linking efficiency of 30%.

From the TofSIMS results, there was a significant increase of 126-fold for glycine and/or amino acid residues $H_2N–CH_2^+$ at *m/z* 28.90. Moreover, the fragmented masses of carboxyl $COOH^+$ and amino acid residues $^+HNCH_2CH_3$ at *m/z* 45.12 showed a similar intensity increase of 147-fold. The large increase in these fragmented masses is suspected to be mainly due to amino acid residues from the fragments of the 9-mer peptide. Other important fragmentation peaks include a 26-fold increase for peptide bonds ($O=C–NH^+$) at *m/z* 43.15, a 3-fold increase for proline residues $C_4H_8N^+$ at *m/z* 70.31, 9-fold increase for leucine fragments $C_5H_{18}N^+$ at *m/z* 85.95, and a 5-fold increase for the terminal amino acid, hydroxyproline $C_5H_{10}O_2N^+$ at *m/z* 131.80. Unfragmented hydroxyproline was identified because it was the terminal amino acid and hence its carboxyl group was intact with the molecule. All other amino acids were difficult to identify as a whole and most of their fragments were incorporated within the amino acid residues (hence the large intensity increases at *m/z* 30.20 and *m/z* 45.12). The identification of the amino acid mass fragments provides a solid foundation to confirm the successful attachment of the BCSP^{TM}-1 peptide onto the surface of Skelite^{TM}.

The functionalized surface in combination with the DSS linkage effectively bound both BSA and BCSP^{TM}-1. However, BSA was bound more readily. This is attributed to the greater number of free amines on the BSA capable of reacting with the DSS on the surface resulting in an increased likelihood of reaction over the time frame used.

Using APTES to functionalize the surface did not render the surface cytotoxic; however, its presence on the Skelite surface, as well as that of bound BCSP^{TM}-1 or BSA, reduced the concentration of adherent cells at day 6. Furthermore, there was no difference between surfaces immobilized with BSA or BCSP^{TM}-1 at day 6; the protein and peptide grafted surfaces showed statistically equivalent cell densities that did not vary with concentration ($p > 0.05$). By day 10, with the exception of the 24 nmol/cm^2 BSA coated surfaces, which had significantly higher cell populations, and the Skelite^{TM}+APTES surfaces, which had significantly lower cell populations, the cell density on all surfaces was equal. The reduction in cell proliferation upon APTES functionalization of the surface is likely due to the increase in surface hydrophobicity, which changes the nature and configuration of protein deposition to the Skelite surface. Unlike RGD containing surfaces, BCSP^{TM}-1 modified surfaces did not exhibit peptide concentration dependent cell adhesion or proliferation. Conversely, BSA modified surfaces did exhibit a surface density dependent increase in cell adhesion and proliferation.

The activity of the BCSP^{TM}-1 bound to the Skelite^{TM} surface was assessed using a fetal rat calvaria cell population and measuring ALP activity/cell. It is important to note that differentiation of osteoprogenitors into osteoblasts is a sequential process, and ALP expression is an early marker of osteoblast differentiation, which ultimately decreases as the cells commit to becoming osteoblasts. Moreover, the rat calvaria culture is a heterogeneous cell population, containing only a small number of osteoprogenitors [27].

Interestingly, the APTES-grafted surface, which had much fewer cells, demonstrated an ALP activity/cell that was significantly higher than the underlying Skelite^{TM} surface ($p < 0.05$) at each time point. This result is similar to that noted by Healy et al. [28] and Davis et al. [7], and is attributed to enhanced, or possibly more specific, protein adsorption to the more hydrophobic Skelite^{TM}-APTES surface, to which the osteoprogenitors in the culture responded.

The ALP activity/cell on the modified Skelite™ was enhanced when the ubiquitous serum protein albumin was bound to the surface compared to the Skelite™ alone at day 6 for both surface densities, but only for the lower BSA surface density of 17 nmol/cm^2 at day 10. Thus, with high concentrations of BSA on the surface, although there were more cells present, fewer cells were of the osteoblastic lineage. Moreover, the relative increase in cellular ALP activity on the albumin bound surface was comparable to that reported by Itoh et al. for RGD physically bound to hydroxyapatite. Itoh et al. report a maximum increase in ALP activity per cell of approximately 45% over the hydroxyapatite alone after 10 days, while the 17 nmol/cm^2 BSA bound surfaces of this work exhibited an increase in cellular ALP activity/cell of 61% over that of Skelite™ at day 10 [12]. It should be noted that the cells used in each case were different, as Itoh et al. employed a murine osteoblastic cell line (KUSA/A1) while primary neonatal rat calvaria osteoprogenitor cells were used in this work. This result suggests that covalently binding albumin to the surface is as, effective at inducing osteoblastic activity on a modified hydroxyapatite than physically binding RGD. However, only one marker for osteoblast differentiation was used for comparison.

Even greater cellular ALP activity per cell was exhibited when low surface densities of BCSP™-1 were bound to the surface. A maximum increase in ALP activity/cell of 276% over that of bare Skelite™ was achieved at a surface density of BCSP™-1 of 0.87 nmol/cm^2 at day 6. A similar concentration response of cellular ALP activity was also noted by Itoh et al. with RGD physically bound to hydroxyapatite [12]. The ALP activity/cell remained essentially constant, or decreased slightly, from day 6 to day 10. This result is consistent with the findings of Declerq et al., who found that the ALP activity/cell of rat calvaria cell cultures in osteogenic medium decreased by approximately 25% from day 4 to day 14 [29]. Our results indicate that, when low concentrations of BCSP™-1 were immobilized to the surface of the Skelite™, there were fewer adherent and proliferating cells present, but a greater number of these cells were of the osteoblastic lineage. The calvaria osteoprogenitor cells were likely responding to the specific amino acid sequence of the collagen type I fragment peptide via an integrin mediated signaling pathway. This response is currently not fully understood, however, it may be attributable to cellular down-regulation of surface integrins in the presence of excess BCSP™-1 stimulation.

Immobilization of the BCSP™-1 on the surface of Skelite™ enabled localization and retention of the peptide at the interface for controlled cell-biomaterial interactions. BCSP™-1 could not be effectively adsorbed to the surface, thus, having the peptide immobilized on the surface is important because the osteoprogenitor cells must interact with the peptide at a desired concentration and for a sufficient duration for maximum cellular activities.

5 Conclusions

This work has demonstrated that the peptide BCSP™-1 was successfully immobilized onto the surface of Skelite™ via the covalent attachment of APTES and DSS linkers. The binding chemistry demonstrated that the Skelite™ surface can be modified via its hydroxyl groups, even though there has been a reduced number of free hydroxyl groups due to the presence of silicon in the crystal lattice. The immobilized peptide was bioactive after immobilization and the chosen chemistry was non-toxic to rat calvaria cells. Although binding of the peptide to the Skelite™ surface resulted in a reduction in cell adhesion and proliferation, Skelite™ surfaces with BCSP™-1 attached stimulate increased ALP activities per cell when employing a rat calvaria primary cell culture. BCSP™-1 covalently attached on the surface of Skelite™ stimulated a roughly 3-fold increase in ALP activity per cell when compared to a non-modified Skelite™ or when compared to BCSP™-1 adsorbed Skelite™ after 6 days of culture. Comparisons were also made to monitor ALP activities on an albumin bound surface versus BCSP™-1 bound surface (given similar surface concentrations in nmol/cm^2) and it was determined that the BCSP™-1 bound surface on average stimulated an increased ALP activity per cell. These results indicate that BCSP™-1 attached to the Skelite™ surface bound a greater number of osteoprogenitor cells from the heterogeneous rat calvaria cell pool. Moreover, the cellular ALP activity was comparable with albumin covalently bound to the surface compared to reports on the binding of RGD sequences to ceramic surfaces via physical means, in support of recent findings that RGD-coated surfaces were not as effective at inducing mesenchymal stem cells to differentiate as serum proteins adsorbed from serum. These findings signify that the chosen methodology to incorporate BCSP™-1 with a ceramic was successful and non-cytotoxic, and that the presence of this peptide layer resulted in osteoprogenitor cell adhesion and progression towards differentiation osteoblasts, which ultimately may lead to improved bone healing in a clinical setting. Future work should explore whether the osteoprogenitors fully differentiate into osteoblasts, as well as determining the mechanism by which BCSP™-1 elicits its effect.

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